Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow\textsuperscript{1,2}

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BERNE, ROBERT M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am. J. Physiol. 204(2): 317-322. 1963.—Experiments were performed on isolated cat hearts perfused with Tyrode's solution and intact hearts of open-chest dogs. Cardiac hypoxia resulted in a decrease in coronary vascular resistance and a release of significant amounts of inosine and hypoxanthine from the myocardium. From 3 to 27 times more inosine and hypoxanthine were released from the heart during myocardial hypoxia than were required to double the coronary blood flow when infused as adenosine into the left coronary artery. Based on the assumption that with hypoxia the nucleotide derivatives leave the myocardial cell as adenosine, an hypothesis is proposed for the metabolic regulation of coronary blood flow.

A constant level of energy expenditure by the myocardium, coronary blood flow is relatively stable in the face of alterations in perfusion pressure \textsuperscript{1,2}. The nature of the mechanism responsible for this phenomenon, known as autoregulation, remains obscure. The cardiac nerves are apparently not involved, since autoregulation is readily observed in the denervated heart. However, the occurrence of reactive hyperemia in the heart and the striking parallelism between coronary blood flow and the metabolic activity of the myocardium have directed attention to products of metabolism as possible mediators. To date, none of the known metabolites or vasoactive constituents of cardiac muscle have been shown to be released from the myocardium in concentrations sufficient to account for the coronary vasodilation observed with hypoxia or augmented cardiac metabolism \textsuperscript{3}. Furthermore, reoxygenated coronary sinus blood collected from hearts subjected to hypoxia or ischemia failed to produce changes in vascular resistance when infused into test coronary vessels \textsuperscript{4}. This observation could mean that if a vasoactive substance is released from the myocardium during hypoxia it is rapidly inactivated and/or greatly diluted by the blood.

Adenosine may conceivably be the vasoactive substance since: \textsuperscript{1} it is a potent coronary vasodilator \textsuperscript{5}; \textsuperscript{2} it is rapidly inactivated by adenosine deaminase in blood and tissues \textsuperscript{6,7}; \textsuperscript{3} it can rapidly penetrate the myocardial cell membrane \textsuperscript{7}; and \textsuperscript{4} there is a large potential source of adenosine in the form of adenosine nucleotide in the myocardium. Analysis of nucleotide derivatives in perfusates and coronary sinus blood was undertaken to test the hypothesis that the release of adenosine from the myocardium is responsible for the coronary vasodilation observed in cardiac hypoxia.

METHODS

Cat heart experiments. Cats were struck a sharp blow on the head and the hearts of the unconscious animals were quickly excised, rinsed with warm Tyrode's solution, and perfused with oxygenated Tyrode's solution in the manner originally described by Langendorff \textsuperscript{8}. The perfusion fluid was equilibrated with \textsuperscript{1} 95% O\textsubscript{2} and \textsuperscript{2} 5% CO\textsubscript{2} and introduced into the aorta at a pressure of 40 cm of water. After all residual blood was flushed out of the heart, a control period was begun in which 200 ml of oxygenated Tyrode's solution was recycled through the heart for 15 min and then collected in an ice bath. In the recovery period the heart was perfused with oxygenated Tyrode's solution and the perfusate collected. The perfusates were then treated with perchloric acid for protein precipitation and the nucleotide derivatives adsorbed on charcoal, eluted, separated on Dowex-I-chloride columns and assayed enzymatically as previously described \textsuperscript{7}.

Two hundred milliliters of this anoxic perfusion fluid was recycled through the heart for 15 min and collected in ice-cold flasks. In the recovery period the heart was perfused with oxygenated Tyrode's solution and the perfusate collected. The perfusates were then treated with perchloric acid for protein precipitation and the nucleotide derivatives adsorbed on charcoal, eluted, separated on Dowex-I-chloride columns and assayed enzymatically as previously described \textsuperscript{7}.

Dog heart experiments. Dogs were anesthetized with intravenously administered sodium pentobarbital (30 mg/kg), placed on artificial respiration, and thoracotomized in the fourth left intercostal space. After the ad-
in isolated perfused cat hearts

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Coronary Flow, ml/min</th>
<th>Heart Rate, beats/min</th>
<th>Hypoxanthine, µg/ml Perfusate</th>
<th>Inosine, µg/ml Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>74</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anoxia</td>
<td>20</td>
<td>29</td>
<td>0.166</td>
<td>0.233</td>
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<tr>
<td>Recovery</td>
<td>9</td>
<td>50</td>
<td>0.098</td>
<td>0.210</td>
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<tr>
<td>Control</td>
<td>14</td>
<td>128</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Anoxia</td>
<td>21</td>
<td>88</td>
<td>0.084</td>
<td>0.250</td>
</tr>
<tr>
<td>Recovery</td>
<td>14</td>
<td>102</td>
<td>0.021</td>
<td>0.062</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>120</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anoxia</td>
<td>32</td>
<td>77</td>
<td>0.028</td>
<td>0.161</td>
</tr>
<tr>
<td>Recovery</td>
<td>17</td>
<td>108</td>
<td>0.028</td>
<td>0.088</td>
</tr>
</tbody>
</table>

RESULTS

Cat heart experiments. Adenine nucleotides and adenosine were not present in the perfusates of hearts perfused with oxygenated or deoxygenated Tyrode's solution. It is quite possible that adenosine was released from the perfused heart but was degraded to inosine and/or hypoxanthine before the addition of perchloric acid to the perfusate. Previous studies (7) have shown that adenosine deaminase and nucleoside phosphorylase, which convert adenosine to inosine and hypoxanthine, are leached out of hearts perfused with oxygenated Tyrode's solution.

Replacement of oxygenated Tyrode's solution with Tyrode's solution equilibrated with nitrogen resulted in the appearance of hypoxanthine and inosine in the perfusate (Table 1). Reinstitution of oxygenated perfusion fluid produced a decrease in the amounts of these compounds released from the heart. Although the absolute values for the hypoxanthine and inosine content of the perfusates are large they represent only about 0.3–0.4% of the total adenine nucleotide content of the myocardium. Coronary vasodilation and a reduction in heart rate were associated with the anoxia-induced appearance of inosine and hypoxanthine. The bradycardia is indicative of the severe degree of anoxia produced in these experiments.

Dog heart experiments. The isolated Tyrode-perfused nonworking cat heart was employed in the initial studies because it is a simple preparation free of possible contaminating nucleotide derivatives from erythrocytes and one which can be subjected to complete anoxia. Gross changes observed with such a preparation may be indicative of normal phenomena but, because of the unphysiological nature of the preparation, extrapolation to normal heart muscle could be misleading. Therefore, the next series of experiments was carried out on the intact working heart in the open-chest dog in order to determine whether similar observations could be made under more physiological conditions. Before undertaking animal experiments, it was considered desirable to demonstrate whether similar observations could be made under more physiological conditions. Before undertaking animal experiments, it was considered desirable to demonstrate whether similar observations could be made under more physiological conditions.

Separation of the nucleotide derivatives was accomplished by paper chromatography, since the use of columns resulted in poor recoveries when small amounts of material eluted with water in a chromatography jar saturated with water vapor. All UV-absorbing material was eluted from the small strips in 1–3 ml water in about 20 hr. Volumes were adjusted to 2.1 ml by evaporation at 40°C or by the addition of water. Aliquots of this solution were then analyzed for their inosine and hypoxanthine content by the enzymatic methods of Kalckar (9) using microcuvettes in the Beckman model DU spectrophotometer. All determinations were made in duplicate.

ministration of heparin, the left coronary artery was cannulated with an Eckstein cannula and the coronary sinus cannulated with a modified Murawicz cannula. The left coronary artery was perfused with blood from the left carotid artery and the coronary sinus outflow was directed to the left jugular vein. Coronary blood flow was measured with a rotameter and mean left coronary perfusion pressure with a mercury manometer. During control periods the animal was respired with room air, whereas during the experimental periods partial asphyxia was produced by greatly reducing the respiratory minute volume. Approximately 30 ml of coronary arterial and coronary sinus blood were simultaneously obtained from the tubing of the respective cannulae. In experimental periods, when asphyxia was well established, coronary blood flow was reduced to control levels by an adjustment of a screw clamp on the coronary artery inflow tubing before blood samples were drawn. The blood was immediately chilled and the red cells sedimented in a refrigerated centrifuge. Fifteen to twenty milligrams of acid-washed, activated wood charcoal (Darco) was added to each plasma sample (ca. 12 ml) and the suspension shaken in a cold room for 60 min. After centrifugation, the plasma was discarded and the sedimented charcoal was transferred with 1.5 ml of water to a column 1 cm in diameter containing 150 mg of washed Celite packed over a small glass-wool plug. Fifty per cent ethanol at pH 10 was put through the column until the eluate read zero against the eluting solution in the Beckman model DU spectrophotometer at wave lengths of 249 and 260 m. The eluate samples were evaporated to dryness at 40°C or by the addition of water. Aliquots of this solution were then analyzed for their inosine and hypoxanthine content by the enzymatic methods of Kalckar (9) using microcuvettes in the Beckman model DU spectrophotometer. All determinations were made in duplicate.

4 In earlier studies plasma proteins were precipitated with perchloric acid and the resultant supernatant treated with charcoal. This procedure was discontinued because of the losses of nucleotide derivatives, presumably due to adsorption on the protein precipitate.

TABLE I. Effect of hypoxia on coronary flow, heart rate, and release of myocardial inosine and hypoxanthine in isolated perfused cat hearts
NUCLEOTIDE DEGRADATION IN MYOCARDIAL HYPOXIA

made detection of these nucleotide derivatives impossible. Inosine or hypoxanthine, it was thought possible that dilution by the large volume of blood perfusing the heart during these experiments revealed insignificant quantities of material used. However, it was necessary to have approximately 10 μmole or more per spot in order to be able to delineate the area on the paper under UV light with relative ease. In these amounts, recoveries from the paper were quantitative. Percentage recoveries of inosine and hypoxanthine from dog plasma were determined at plasma concentrations of 1-6 μmole/ml plasma in 28 trials using 6 different plasma samples. At these concentrations the per cent recovery of added inosine was 83.2, with a standard deviation of 13.0, and for added hypoxanthine was 80.9, with a standard deviation of 16.7. Recoveries from dog plasma previously treated with charcoal to remove all detectable amounts of inosine and hypoxanthine were equally good at the lowest and highest concentrations studied. However, when endogenous inosine or hypoxanthine was present in the plasma used for recovery studies, calculation of the added amounts of these compounds by the difference between the total and the endogenous contents gave unreliable results at the lowest concentrations.

To avoid the need of blood replacement, arterial and venous blood samples did not exceed 30 ml. This limitation of the size of the blood sample meant that plasma concentrations of much less than 1 μmole/ml plasma could not be accurately determined because of weak UV absorbance on the paper chromatogram and borderline changes in optical density in the enzymatic assay.

In the first few experiments no attempt was made to restrict coronary blood flow during the periods of partial asphyxia. Consequently, a large flow increase occurred as a result of the hypoxemia and the increase in aortic pressure which usually accompanied the partial asphyxia. Since analysis of coronary sinus blood in all but one of these experiments revealed insignificant quantities of inosine or hypoxanthine, it was thought possible that dilution by the large volume of blood perfusing the heart made detection of these nucleotide derivatives impossible.

The data from the one experiment in which significant quantities of hypoxanthine were present in coronary sinus blood (with asphyxia), despite a twofold increase in coronary blood flow, are shown in Table 2 (exp. 3). A second period of asphyxia, more severe than the first, was associated with an increase in the concentration of hypoxanthine in the coronary sinus blood and the appearance of significant amounts of inosine. In the remaining experiments presented in Table 2, coronary blood flow was adjusted to approximately control levels during the asphyxia period by means of a screw clamp on the arterial inflow tubing. As can be seen from the reduction in perfusion pressure required to maintain a relatively constant coronary blood flow, vascular resistance was significantly reduced by the partial asphyxia. On release of the constriction and restoration of perfusion pressure to approximately control levels, coronary blood flow showed a large overshoot above control.

During each of the hypoxic periods of the last four experiments in Table 2, the coronary sinus plasma fraction contained 4.4-19.2 μmole hypoxanthine and 1.0-15.8 μmole inosine/ml plasma, whereas simultaneously drawn arterial blood samples contained 0.0-0.4 μmole hypoxanthine/ml plasma and no inosine. In control and recovery arterial and venous plasma samples inosine was absent and the maximum concentration of hypoxanthine found was 0.7 μmole/ml.

An approximation of the amounts of nucleotide derivatives released per minute from the heart during the periods of asphyxia is presented in Table 2. These estimates are products of the coronary blood flow in milliliters per gram wet weight of heart and the sum of the arteriovenous differences for inosine and hypoxanthine. The values ranged from 2.7 to 21.3 μmole/min g heart.

Glutamic oxalacetic transaminase determinations were carried out on control and experimental arterial and
were 0.84 and 0.80 for experiments g and h with the rate of release in asphyxia (Table 3) was 5.3 and 3.7 μmoles/ml plasma, respectively. The amounts released were approximately one-half of the expected concentration of inosine in the arterial blood plasma samples during experimental or control periods. Also adenosine deaminase was not found in control or asphyxia arterial or venous blood samples.

In two experiments adenosine was infused at a rate of 0.25 μmoles/min into the tubing leading to the left coronary artery at a point proximal to the rotameter (Table 3). This amount of adenosine produced a two-fold increase in coronary blood flow which was returned to approximately control levels by compression of the inflow tubing prior to simultaneous arterial and venous sampling. Complete mixing of the infused adenosine with the arterial blood had occurred by the time the blood reached the point where arterial samples were drawn. Analysis of coronary arterial and venous blood samples revealed the absence of hypoxanthine and the presence of inosine in the arterial blood. In these experiments any adenosine would appear as inosine, since no attempt was made to separate the two nucleosides, and adenosine deaminase was present in the myocardium. * Includes adenosine since no attempt was made to separate the two nucleosides. No hypoxanthine was found. † Adenosine dissolved in 0.9% NaCl infused at 0.25 μmoles/min into left coronary artery.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Perfusion Pressure, mm Hg</th>
<th>Coronary Blood Flow, ml/min</th>
<th>Inosine, μmoles/ml Plasma</th>
<th>Inosine, μmoles/min/g Heart</th>
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<tr>
<td>Control</td>
<td>84</td>
<td>59</td>
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<td>Adenosine†</td>
<td>76</td>
<td>124</td>
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<td>Adenosine with release of constriction</td>
<td>82</td>
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<tr>
<td>Recovery</td>
<td>03</td>
<td>64</td>
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Experiment g (163)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Perfusion Pressure, mm Hg</th>
<th>Coronary Blood Flow, ml/min</th>
<th>Inosine, μmoles/ml Plasma</th>
<th>Inosine, μmoles/min/g Heart</th>
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<tr>
<td>Control</td>
<td>92</td>
<td>71</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenosine†</td>
<td>88</td>
<td>128</td>
<td>1.9</td>
<td>0.80</td>
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<tr>
<td>Adenosine with constriction of tubing</td>
<td>51</td>
<td>71</td>
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<td>0.80</td>
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<tr>
<td>Adenosine with release of constriction</td>
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<tr>
<td>Recovery</td>
<td>88</td>
<td>68</td>
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</table>

Experiment 10 (162)

Numbers in parentheses refer to wet weight of perfused myocardium. * Includes adenosine since no attempt was made to separate the two nucleosides. No hypoxanthine was found. † Adenosine dissolved in 0.9% NaCl infused at 0.25 μmoles/min into left coronary artery.

Failure to detect any inosine or hypoxanthine in coronary sinus blood during intracoronary artery infusion of adenosine is consonant with a previous observation that adenosine readily enters myocardial cells where it is phosphorylated to adenosine triphosphate (ATP) (7).

DISCUSSION

With hypoxia, inosine and hypoxanthine appeared in the perfusate of the isolated cat heart and in the coronary sinus blood of the intact, working dog heart. Neither of these adenine nucleotide derivatives was present in significant amounts in arterial blood of the hypoxic dog or in arterial or cardiac venous blood of the normally ventilated animal. These observations indicate that the inosine and hypoxanthine originated in the myocardium and that the partial asphyxia in the dog did not produce a release of such compounds from other body tissues in quantities sufficient to be detected in the arterial blood. Because of its simplicity the Langendorff preparation was ideally suited for preliminary studies. However, the release of inosine and hypoxanthine from the anoxic cat heart may not be physiologically significant, since the heart is perfused with salt solution and is somewhat hypoxic even during control periods, as suggested by the leakage of enzymes into the perfusion fluid (7). In contrast, the hypoxic dog heart is capable of performing work and shows no impairment of the integrity of the cell membranes, as evidenced by the absence of transaminase or adenosine deaminase in coronary sinus blood. However, the presence of erythrocytes in the dog heart experiments poses several problems. The erythrocytes are rich in ATP and thus are a potential source of contaminating adenine nucleotide derivatives. Therefore, it is necessary to exercise considerable care in the collection and handling of the blood samples to avoid red cell lysis. A second problem is the uptake and metabolism of nucleosides by the red cells. Inosine and adenosine readily enter red cells where the ribose moiety is metabolized and the purine can be utilized in the synthesis of nucleotides. The experiments on the intracoronary infusion of adenosine (Table 3) indicate that approximately one half of the nucleoside disappeared between the point of infusion and the point of sampling (transit time between these points being about 15 sec). Presumably the lost nucleoside was in the erythrocytes. A third problem related to the use of blood as a perfusion medium is the presence of adenosine deaminase and nucleoside phosphorylase in erythrocytes. These enzymes rapidly degrade adenosine and thereby make accurate calculation of the effective concentration of adenosine at its site of action virtually impossible.

The amount of the nucleotide derivatives released per minute from the hypoxic dog heart constitutes a small fraction (approx. 1/200 to 1/2,000) of the available adenine nucleotide, in contrast to complete anoxia in which the total adenine nucleotide content of the intact heart is reduced to about 60% of normal in a period of 45 min (10). However, if the hypoxic period was to persist for more
NUCLEOTIDE DEGRADATION IN MYOCARDIAL HYPOXIA

necessary to produce a marked reduction in coronary re-
fraction of the amount recovered in the present study is
leased from the myocardium as adenosine, only a small
would be lost from the heart, particularly with a severe
from the heart during myocardial hypoxia than are re-
cardium perfused per unit time, one finds that from 3 to
NUCLEOTIDE DEGRADATION IN MYOCARDIAL HYPOXIA

In none of the cat or dog experiments were adenosine
or the adenosine nucleotides detected in the heart effluents.
There is no good evidence that the phosphorylated
adenosine compounds can traverse the intact cell mem-
brane, although adenosine has been shown to enter myo-
cardial cells with relative ease (7). Adenosine and the
adenine nucleotides are potent vasodilators, whereas
inosine and hypoxanthine are virtually ineffective in
altering vascular resistance. To be of physiological signi-
cance in the regulation of coronary blood flow it must be
demonstrated that the inosine and hypoxanthine leave
the myocardial cell in a form that has vasodilator ac-
tivity. At present it has not been possible to show that
any of the recoverable nucleotide derivatives cross the
myocardial cell membrane as adenosine. In the cat heart
experiments adenosine deaminase and nucleoside phos-
phorylase are leached out of the perfused heart and any
adenosine released into the perfusate would be converted
to inosine and hypoxanthine before in vitro inactivation
of these enzymes could be accomplished (7). In the dog-
heart experiments the time required to collect the coro-
nary sinus blood and to separate cells and plasma is suffi-
cient for complete destruction of any adenosine which
might have been present.

Studies by Angkapindu, Stafford, and Thorp (11) on
aqueous extracts of guinea pig heart suggest that de-
phosphorylation of adenylic acid (AMP) precedes
deamination. If this is true in the intact heart it is con-
ceiveable that the inosine found in the heart effluents is in
some measure derived directly from adenosine by deami-
nation and not by the dephosphorylation of inosinic acid.
Based on the assumption that a significant amount of the
adenosine formed from AMP is deaminated outside of
the myocardial cell membrane, the following hypothesis for
the metabolic regulation of coronary blood flow is proposed
(Fig. 1). In this scheme the key factor is the myocardial
oxgen tension. Reduction in myocardial oxygen tension
by hypoxemia, decreased coronary blood flow, or in-
creased oxygen utilization by the myocardial cell leads to
the breakdown of heart muscle adenine nucleotides to
adenosine. The adenosine diffuses out of the cell and
reaches the coronary arterioles via the interstitial fluid
and produces arteriolar dilation. The resultant increase
in coronary blood flow elevates myocardial oxygen
tension, thereby reducing the rate of degradation of
adenine nucleotides, and decreases the interstitial fluid
concentration of adenosine by washout and enzymatic
destruction. This feedback mechanism serves to adjust
coronary blood flow to meet the new metabolic require-
ments and a new steady state is reached. Restoration of
normal myocardial oxygen tension prevents the deg-
radation of adenine nucleotides and the formation of

than several minutes, considerable adenine nucleotide
would be lost from the heart, particularly with a severe
degree of hypoxia. In the present experiments it was
necessary to produce severe hypoxia in order to demon-
strate convincingly that nucleotides could be released
from a functioning heart. However, with more physi-
ological stimuli, much smaller and yet effective vaso-
dilator quantities of nucleotide derivatives may be re-
leased from the myocardium. It is evident from Table 3
that very small and probably undetectable concentra-
tions of adenosine in arterial blood are effective in elic-
iting a vasodilator response in the coronary vessels. First,
one-half of the adenosine disappeared between the point
of infusion and the point of sampling of arterial blood,
making it unavailable as a vasodilating agent. Second, a
large portion of the recoverable nucleoside had probably
been inactivated by deamination during the brief ex-
poure to the blood, since Conway and Cooke (6) have
shown that adenosine is deaminated in shed blood in a
matter of seconds. It is quite reasonable to expect that
the concentration of adenosine that reached the effective
site, the coronary arterioles, was still further reduced by
virtue of continuing deamination and utilization by the
red cells as the blood containing adenosine coursed
through the cannula and coronary arteries. Consider-
ation of these points, in the light of the barely detectable
concentration of adenosine found in coronary arterial
flow during infusions of adenosine at a rate which elicited a
twofold increment in coronary blood flow (Table 3), empha-
sizes the vasodilator potency of this nucleoside. Furthmore, when the infused adenosine
(Table 3) and the nucleotide derivatives released from
the heart during moderate to severe asphyxia (Table 2)
are compared on the basis of equal amounts of myo-
cardium perfused per unit time, one finds that from 3 to
27 times more inosine and hypoxanthine are released
from the heart during myocardial hypoxia than are re-
quired to double the coronary blood flow when infused as
adenosine into the left coronary artery. These calcula-
tions indicate that if the nucleotide derivatives are re-
leased from the myocardium as adenosine, only a small
fraction of the amount recovered in the present study is
necessary to produce a marked reduction in coronary re-

\[ \text{Coronary Blood Flow} \rightarrow \text{Myocardial Metabolism} \]

\[ \text{Intracellular Adenine Nucleotides} \rightarrow \text{Extracellular Fluid Adenosine} \]

\[ \text{Washout} \rightarrow \text{Diameter Coronary Arterioles} \]
adenosine, and permits the intrinsic vascular tone to reduce coronary blood flow to control levels.

REFERENCES


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